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HIV ANTIGENS FOR DISEASE INTERVENTION

MIDTERM REPORT

TUN-HOU LEE

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13. ABSTRACT (Maximum 200 words) HIV-1 is the etiologic agent of AIDS. The outer envelope glycoprotein of HIV-1 is more than half sugar, with an average of 24 N-linked glycosylation sites. The sugar mass may provide a form of immune masking. In this effort, we evaluated, by site directed mutagenesis, the relative importance of each of the 24 N-linked glycosylation sites of gp120 in the molecular clone HXB2 in terms of viral infectivity. We found that most of the individual consensus N-linked glycosylation sites are dispensable for activity, and that the five consensus N-linked sites that are likely to have important roles in infectivity are all located in the amino terminal half of gp120, indicating that the N-linked glycosylation sites important for infectivity are not randomly distributed in gp120. In comparing the N-linked glycosylation sites of the N-terminal and C-terminal region of gp120, combination glycosylation site mutants were generated. While N-linked oligosaccharides at the C-terminus were not critical the removal of three or more N-linked glycosylation sites from the N-terminal region affected envelope processing and viral infectivity. The conservation of carbohydrate moieties at the C-terminus of gp120 may be an evolutionary response for HIV to escape immune masking. Based on this, we believe that gp 120 molecules that lack some of				
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the N-linked glycosylation sites in the C-terminal half will be better candidates for vaccines.

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INTRODUCTION:

Human Immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immune deficiency syndrome (AIDS). The HIV-1 *Env* gene encodes a 160 KD glycoprotein which is subsequently cleaved into two smaller species, the extracellular protein gp120 and the transmembrane protein gp41. HIV-1 binds to the CD4 receptor. The CD4 binding domain has been mapped to the C-terminal of gp120, although the N-terminal of gp120 may also be involved in CD4 binding. CD4 depletion is a characteristic phenomenon of AIDS. Since envelope glycoprotein is involved in the first step of HIV-1 and CD4 interaction, vaccine development has been focused here. Our approach to the design of a vaccine has included selectively modifying the HIV envelope by site directed mutagenesis. The HIV envelope may contain immunodominant epitopes that serve as immune decoys and elicit largely non-protective antibodies. We believe that gp120 molecules that lack the most immunodominant epitopes will be better vaccine candidates. In this part of the reporting period we have concentrated on the N-linked glycosylation sites of the envelope outercoat, and we have compared their importance for viral infectivity. We have conducted two studies to address this issue.

More than 20 consensus N-linked glycosylation sites occur in the gp120 coding sequence of most HIV-1 isolates. Based on the N-linked glycosylation pattern of a well-characterized recombinant gp120, it is likely that N-linked sugars are present at most, if not all, of the consensus glycosylation sites of the heavily glycosylated gp120. In the first study, we evaluated the relative importance of each of the 24 N-linked glycosylation sites of gp120 in the molecular clone HXB2 to viral infectivity. The ability of HXB2-derived mutants, each having one of the twenty-four N-linked glycosylation sites mutated by site-directed mutagenesis, to infect CD4-positive SupT1 cells was compared with that of the wild type virus. We found that most of the individual consensus N-linked glycosylation sites are dispensable for viral infectivity. The five consensus N-linked glycosylation sites that are likely to have important roles in HIV-1 infectivity are all located in the N-terminal half of gp120, indicating that the N-linked glycosylation sites that are important for HIV-1 infectivity are not randomly distributed in gp120.

The results of this study indicate that each N-linked glycosylation site is not equally important for biologic activity. Previous studies have demonstrated the possibility of simultaneously removing specific N-linked glycosylation sites without affecting the biologic function. Based on this, nine combination mutants were generated in the N-terminal and C-terminal region and their effects on cleavage of gp160 to gp120 and viral infectivity were assayed. In this study, we generated N-linked glycosylation site combination mutants at the cys 119-205 region of the N-terminal of gp120. These mutants have two, three, four, and five mutated N-linked glycosylation sites (designated N2, N3, N4, and N5, respectively) and combination mutants

at the C-terminal of gp120 which have two, three, four, five, and six mutated N-linked glycosylation sites (C2, C3, C4, C5, and C6, respectively). We compared the effects of these mutations on envelope processing and viral infectivity. Mutants N2, C2, C3, C4, C5, and C6 do not affect gp160 cleavage and can infect CD4-positive T cell lines and form syncytia. However, mutants N3, N4, and N5 lost their ability to infect CD4-positive T cell lines. The impairment of viral infectivity of mutants N3, N4, and N5 can be attributed to inefficient envelope processing. We conclude that N-linked oligosaccharides at the C-terminal of gp120 are not critical for envelope processing, viral infectivity and syncytium formation. However, removal of more than three N-linked glycosylation sites (amino acids 141, 160, and 186) from the cys 131-196 region of the N-terminal of gp120 affects envelope processing and viral infectivity.

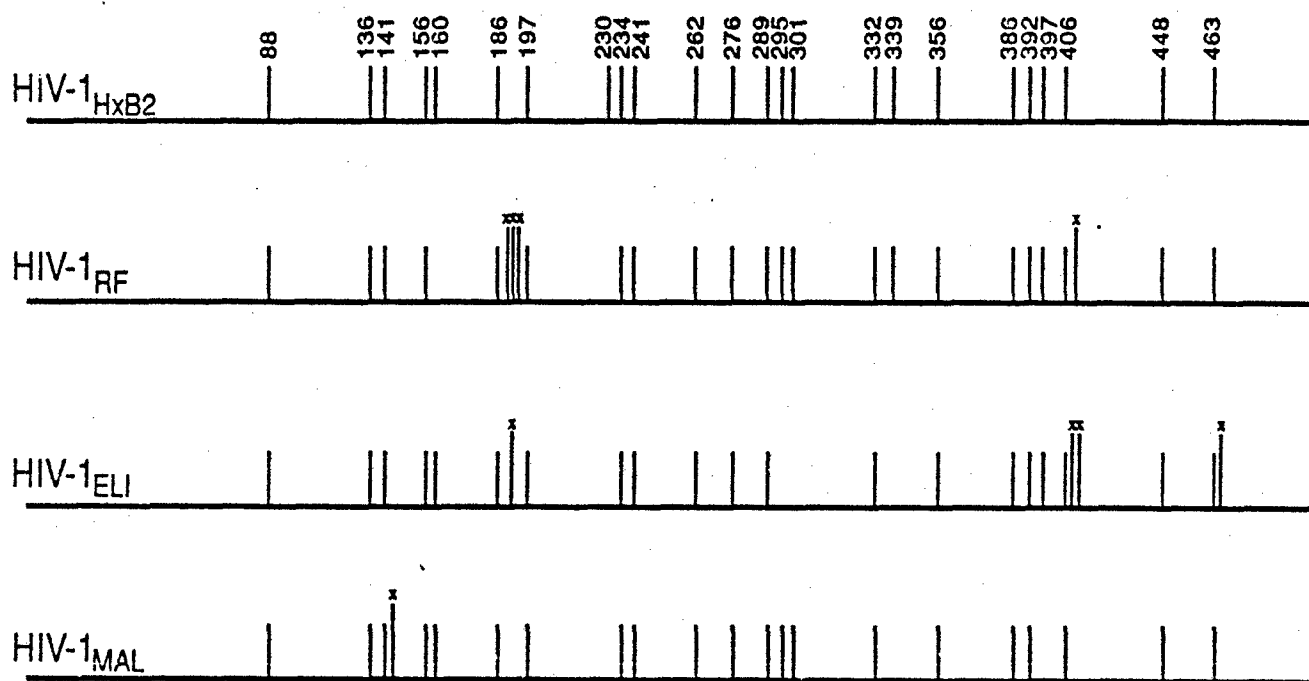
Section 1. Non-random distribution of gp120 N-linked glycosylation sites important for HIV-1 infectivity

The coding region of gp120 is the most divergent among HIV-1 genes (1,2,10,11,29,31,34,36,37). Despite the sequence divergence, at least 20 consensus N-linked glycosylation sequences (Asn-X-Ser/Thr) occur in the gp120 coding region of most HIV-1 isolates (37); about half of these consensus N-linked glycosylation sequences are highly conserved as they occur in identical positions of the various gp120 molecules (Fig. 1). The remaining consensus N-linked glycosylation sequences often occur in the same region of gp120 suggesting a high degree of conservation of these sequences as well (37). Leonard *et al.* reported that N-linked sugars were present in all 24 consensus N-linked glycosylation sites of a recombinant gp120 molecule (17) which suggests that most, if not all, of the potential N-linked glycosylation sites of HIV-1 gp120 contain N-linked sugars.

It is now well-established that gp120 is a heavily glycosylated protein with approximately half of its mass contributed by carbohydrate moieties (8,17,24,38). This is supported by several studies which show that endoglycosidase-treated gp120 migrates as a protein of about 60 Kd, which is in close agreement with the predicted size of an unglycosylated gp120. The gp120 molecules isolated from different sources were found to contain primarily, if not exclusively, N-linked sugars of the high mannose type, the complex type, and the hybrid type (8,17,24). Previous studies using inhibitors of trimming glycosidases involved in the pathway of N-linked glycosylation suggested that these three types of N-linked sugars might not have equally important roles in syncytium-formation and HIV-1 infectivity. For instance, the ability of HIV-1 to form syncytia was affected by deoxynojirimycin, an inhibitor of glucosidase I (39-42), but not by deoxymannojirimycin, an inhibitor of Golgi α -mannosidase I (39,42). These observations suggested that the formation of complex and hybrid types of N-linked sugars were not critical for syncytium formation by HIV-1, because the synthesis of the precursor for these two types of N-linked sugars requires Golgi α -mannosidase I.

For HIV-1 infectivity, blocking the formation of the hybrid type of N-linked sugars or its high mannose (Man₅₋₈) precursors appears to be most critical. Montefiori *et al.* and Pal *et al.* reported that HIV-1 grown in cultures treated with deoxymannojirimycin had reduced infectivity (41,42). Such findings could suggest that the synthesis of complex or hybrid types of N-linked sugars or their immediate Man₅₋₈ precursors are critical for HIV-1 infectivity. However, based on the finding that swansonine, which inhibits Golgi α -mannosidase II and prevents the synthesis of complex type sugars, had no effect on viral infectivity (42), the complex type of N-linked sugar is unlikely to have a significant role in viral infectivity.

Figure 1 Conservation of consensus N-linked sites in HIV-1 gp120. 24 N-linked glycosylation sites of HXB2 are shown. The numbers above each line indicate the amino acid positions in HXB2. The longer lines with an asterisk symbol represent N-linked glycosylation sites not present in HXB2.



While the syncytium-forming ability of HIV-1 and viral infectivity are clearly influenced by N-linked sugars, the global action of inhibitors of trimming glycosidases makes it difficult to assess the relative importance of the individual N-linked glycosylation sites in the gp120 molecule. The present study was designed to address the questions of whether N-linked glycosylation sites present in some parts of the gp120 molecule are more critical for viral infectivity than others and whether the removal of a particular N-linked glycosylation site affects HIV-1 infectivity.

Construction of N-linked glycosylation site mutants. Oligonucleotide-directed mutagenesis was performed on a 2.7 Kb Sal I-BamH I fragment of HXB2 (6), which covers all 24 N-linked glycosylation sites of gp120, using the method of Kunkel (13). The oligonucleotide primers used for mutagenesis were synthesized using standard cyanoethyl phosphoramidite chemistry and are listed in Table I. Mutants were identified by the Sanger chain termination method (27). The Sal-I-BamH-I fragment containing the desired mutation was excised from the replicative form of each mutant and used to replace the 2.7 Kb Sal I-BamH I fragment of HXB2. All HXB2-derived N-linked glycosylation site mutants containing the designated changes were further verified by DNA sequencing (27).

Western blot analysis of envelope proteins. Ten micrograms of wild type or mutant DNA were transfected to 3×10^6 COS-1 cells by the previously described DEAE-dextran method (43). Cell lysates were collected 48 hours after transfection. Mock-transfected, wild type, and mutant transfected COS-1 cells were washed with phosphate-buffered saline (PBS) once and subjected to centrifugation at 2500 rpms. Cell pellets were resuspended with 100 ml RIPA lysis buffer (0.15 M NaCl/0.05 M Tris HCl pH 7.2, 1% Triton X-100, 1% Sodium deoxycholate DOC, 0.1% SDS) and spun down at 35,000 rpm (Ti70 rotor; Beckman) at 4°C for 40 minutes. Ten microliters of cell lysates were electrophoresed in 12% SDS-polyacrylamide gel. A reference HIV-1 positive serum at a 1:200 dilution and a sheep anti-gp120 (AIDS Research Reference Reagent Program #288) at 1:2000 dilution were used for Western blots as previously described (4).

Monitoring of syncytium-formation and viral infectivity. The CD4 positive human T lymphoid cell line, SupT1, was grown and maintained at 37°C in RPMI-1640 containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. COS-1 cells were propagated in Dulbecco minimal eagle medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. Cell-free supernatants were collected 48 hours after transfection. Supernatants were filtered through 0.45mm filters and assayed for virion-associated reverse transcriptase (RT) activity. Equal amounts of wild type and mutant virus, as measured by RT (100K cpm), was used to infect 1×10^6 SupT1 cells. One milliliter of the culture medium was collected every three or four days for RT assay. The culture was monitored for 28 days.

Table 1. N-linked Glycosylation Mutants of HXB2 Envelope Glycoprotein

MUTANT VIRUS INFECTIVITY	AMINO ACID CHANGE	MUTAGENIC OLIGONUCLEOTIDE (5' to 3')*	gp120/160 SIZE CHANGE	VIRAL
88	Asn to Gln	TAGTATTGGT <u>A</u> CGGTGACAGAAAATTT	+	+***
136	Asn to Gln	TGATTTGAAG <u>C</u> AGGATACTAATAC	+	+
141	Asn to Gln	ATACTAATAC <u>C</u> AAAGTAGTAGCGGGA	+	+***
156	Asn to Gln	GATAAA <u>C</u> AGTGCTCTTTCAATAT	+	+
160	Asn to Gln	CTGCTCTTT <u>C</u> AGATCAGCACAAG	+	+***
186	Asn to Gln	TACCAATAGAT <u>C</u> AGGATACTACCAGC	+	+
197	Asn to Gln	TGACAAGTTGT <u>C</u> AGACCTCAGTCAT	+	+***
230	Asn to His	TAA AATGTAAT <u>C</u> ATAAGACGTTCA	+	+
234	Asn to His	ATAAGACGTT <u>C</u> ATGGAACAGGACCA	+	+
241	Asn to Gln	GACCATGTAC <u>A</u> CGGTCAGCACAGTAC	+	+
262	Asn to Gln	ACTGCTGTTA <u>C</u> AAGGCAGTCTAG	+	+***
276	Asn to Gln	TTAGATCTGT <u>C</u> AGTTACGGACAAT	-**	+***
289	Asn to Gln	TAGTACAGCTG <u>C</u> AGACATCTGTAGAAA	+	+
295	Asn to Gln	CTGTAGAAATT <u>C</u> AATGTACAAGAC	+	+
301	Asn to His	ACAAGACCCA <u>C</u> ACAATACAAGAAA	+	+
332	Asn to His	GCACATTGT <u>C</u> ACATTAGTAGAGC	+	+
339	Asn to Gln	GCAAAATGGCAGAACTTTAAAAAC	+	+
356	Asn to Gln	ATTCGGAAAT <u>C</u> AGAAAACAATAATCTTA	+	+
386	Asn to Gln	TTTCTACTGT <u>C</u> AGTCAACACAACCTG	+	+
392	Asn to Gln	ACAACTGTTT <u>C</u> AGAGTACTTGGTTTAATAG	+	+
397	Asn to Gln	GTACTTGGTTT <u>C</u> AGAGTACTTGGAG	-**	+
406	Asn to His	CTGAAGGGTC <u>A</u> ATAA <u>C</u> ACTGAAGGA	+	+
448	Asn to Gln	GATGTTTCATC <u>A</u> AGATTACAGGGCTG	+	+
463	Asn to His	GGTAATAGCA <u>C</u> ATGAGTCCGAGAT	+	+

*Underlined type indicates mutation sites

**Insignificant size change

***Partial impairment

Reverse transcriptase assay. One milliliter of culture medium was mixed with 0.5 ml 30% PEG and 4M NaCl on ice for 2 hours and spun at 2500 rpm at 4°C for 30 minutes. The pellet was resuspended in 100 ml of RT buffer (0.5 % Triton X-100, 15 mM Tris pH 7.4, 3 mM dithiothreitol, 500 mM KCL, 30 % glycerol). Ten microliters of the solution was incubated with 90 ml of RT cocktail (40 mM Tris HCl pH7.8, 10 mM MgCl₂, 8mM dithiothreitol, 94 ml ddH₂O, 0.4 U Poly (rA) oligo (dT) [optical density at 260 nm] per ml and 2.5 mCi/ml ³H-labeled dTTP)

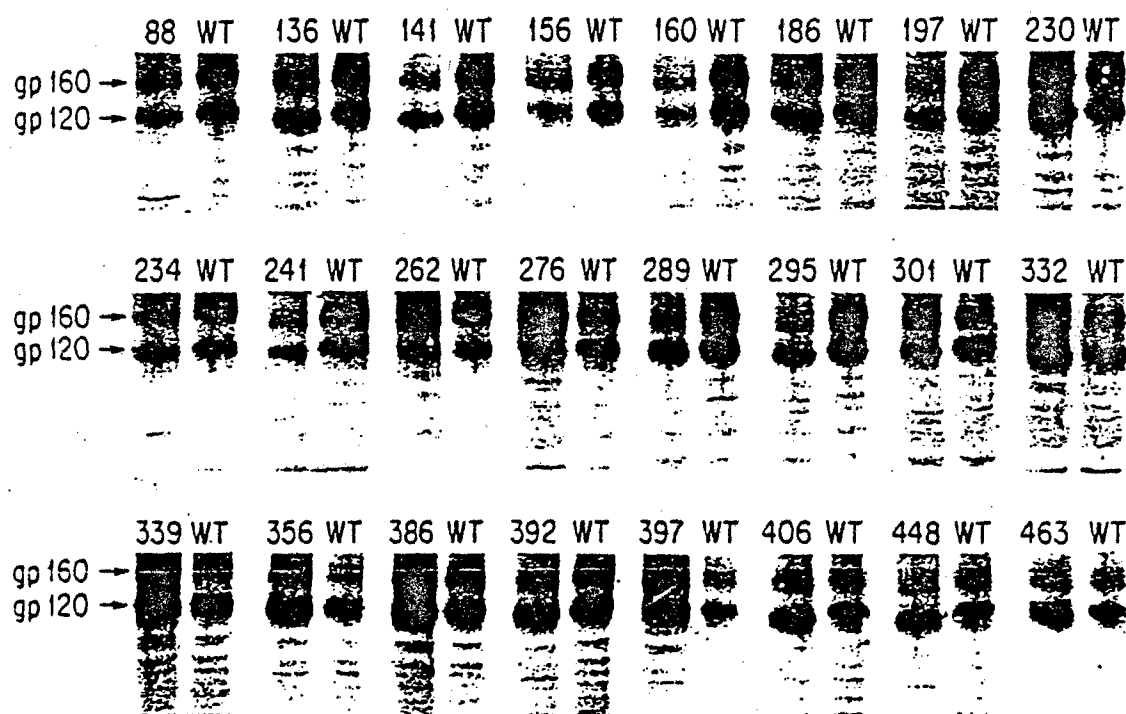
at 37°C for 1.5 hours. The reaction mixture was precipitated with 3ml of 10% trichloroacetic acid (TCA) with 10 ml of 1% tRNA as the carrier and was then chilled on ice for 20 minutes. The reaction mixture was filtered through Whatman GF/C glass microfiber filters and washed 3 times with 5% TCA to remove unincorporated ^3H -dTTP. Radioactivity was quantified in a liquid scintillation counter.

RESULTS:

N-linked glycosylation site mutants. Each of the 24 potential N-linked glycosylation sites in the gp120 coding region of the infectious molecular clone, HXB2, was individually modified to generate 24 N-linked glycosylation site mutants (Table 1). In these mutants, the canonical Asn-X-Ser/Thr sequence was replaced by either Gln-X-Ser/Thr or His-X-Ser/Thr. The underlying hypothesis was that if a given N-linked glycosylation site played no significant role in syncytium-formation or viral infectivity, then such a mutant should retain its ability to form syncytia or remain infectious. Each of the 24 mutants was designated by the residue number of the respective N-linked glycosylation site (Table 1).

Expression of envelope proteins. To determine if mutations introduced to any of the 24 N-linked glycosylation sites affected the expression of the envelope protein, 10 mg each of mutant or wild type proviral DNA were transfected into $3\text{--}5 \times 10^6$ COS-1 cells using the DEAE-dextran method (43). Cell lysates derived from COS-1 transfectants were then subjected to Western Blot analysis. As shown in Figure 2, both gp160 and gp120 were detected in all 24 mutants, suggesting that no particular individual N-linked glycosylation site was indispensable for the expression of the HXB2 envelope protein. Among the 24 mutants, 22 had detectable changes in the migration pattern of gp160 and gp120 (Fig. 1 and Table 1). Such a change could be expected if N-linked sugars were present at those 22 consensus N-linked glycosylation sites of the wild type virus.

Figure 2. Expression of gp160 and gp120 in COS-1 cells transfected with wild-type or mutant proviral DNA. Cell lysates from transfected cells were electrophoresed in SDS/12% polyacrylamide gels, transferred to nitrocellulose filters, and reacted with a reference sheep anti-gp120 serum. The wild-type virus is abbreviated WT, and N-linked glycosylation mutants are indicated by numbers representing their position in HXB2.



Syncytium-formation and viral infectivity. To evaluate whether mutations introduced into any of the individual N-linked glycosylation sites affected syncytium-formation and viral infectivity, cell-free virions from the culture supernatant of COS-1 transfectants were collected 48 hours post-transfection. Equal amounts of mutant and wild type viruses, as measured by reverse transcriptase (RT) activity, were used to infect CD4-positive SupT1 cells. Virus-infected cultures were monitored for syncytium formation and RT activity. As in the case of the wild type virus-infected SupT1 cultures, syncytia and RT activity were detected in all the mutant virus-infected SupT1 cultures (Table 1). However, 6 mutant viruses, 88, 141, 160, 197, 262 and 276, showed delays in growth kinetics when compared with the wild type virus (Table 1). Among the six, mutants 88, 141, 160, and 276 showed similar growth kinetics. The RT activity of the cultures infected by these mutants did not begin to rise until eight days post-infection, lagging behind the wild type virus infected culture by about four days. Mutants 197 and 262 had similar growth kinetics and the RT activity of cultures infected by these mutants did not begin to rise until 16 days post-infection. Figure 3 shows representative growth kinetics of these mutants.

Third-site N-linked glycosylation mutants. To study whether the observed effect on viral infectivity for mutants 88, 141, 160, 197, 262 and 276 was due to amino acid substitutions introduced to replace the asparagine residue of the canonical N-linked glycosylation sequence with a non-canonical residue, six third-site N-linked glycosylation mutants were constructed (Table 2). These six mutants, designated 90, 143, 162, 199, 264 and 278, are called third-site mutants because they had the Ser/Thr residue of the canonical Asn-X-Ser/Thr sequence replaced by a non-canonical amino acid residue. By Western blot analysis, all six third-site mutants had detectable changes in the migration pattern of gp160 and gp120 (Table 2), again suggesting that N-linked sugars may be present at these six consensus N-linked glycosylation sites of the wild type virus.

The ability of these six third-site mutants to infect CD4-positive SupT1 cells was also evaluated. If the phenotype of a third-site N-linked glycosylation mutant is similar to that of the wild type virus, it is likely that the observed defect in infectivity for the corresponding first-site mutant is the result of amino acid substitution at the first site rather than the loss of that particular N-linked glycosylation site. For instance, mutant 162 was indeed found to have similar growth kinetics to the wild type virus (Table 2). This suggested that the impairment of viral infectivity observed for mutant 160 in SupT1 cells was likely due to the substitution of the asparagine residue with a glutamine residue at this particular consensus N-linked glycosylation site; but not due to the loss of this particular consensus N-linked glycosylation site. The remaining five third-site N-linked glycosylation mutants, like their respective first-site mutants, all showed partial impairment in infectivity when compared with the wild type virus (Table 2).

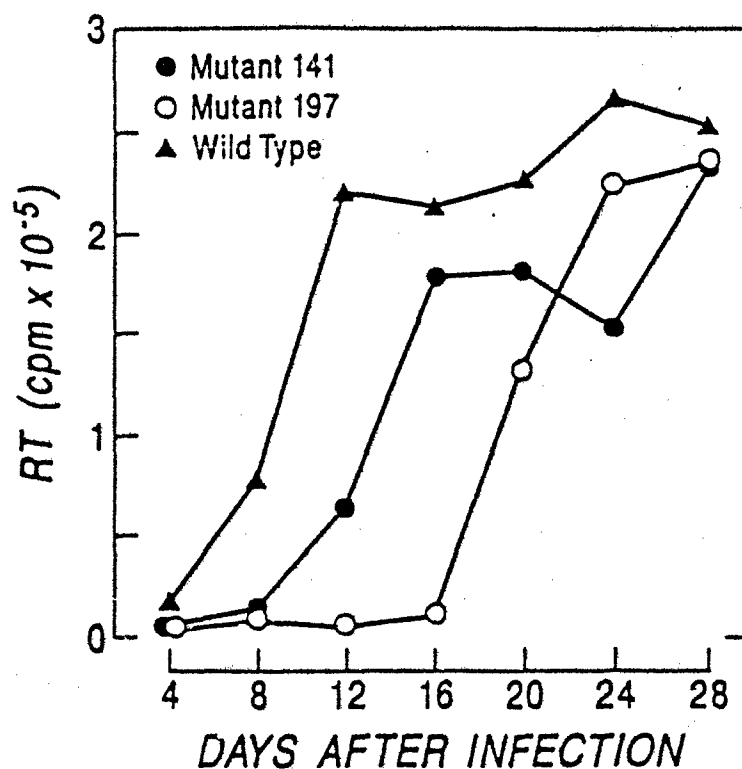
TABLE 2. Third-site N-linked glycosylation mutants of HXB2 Env glycoprotein

MUTANT VIRUS	AMINO ACID CHANGE	MUTAGENIC OLIGONUCLEOTIDE (5' to 3')*	GP120/160 SIZECHANGE	VIRAL INFECTIVITY
90	Thv to Val	GGTAAATGTGG <u>TCGACA</u> ACTTTTGACATGT	+	+++
143	Ser to Ala	AATACCAATAGTGCATGCGGGAGAATGG	+	+++
162	Ser to Ala	CTGCTCTTTCAATATTGCCACAAGCATAAG	+	+
199	Thv to Glu	GTTGTAACACCGAAGTCATTACACAG	+	+++
264	Ser to Ala	CTGCTGTAAATGGCGCTCTAGCAGAAGAAGAG	+	+++
278	Thv to Val	CTGTCAATTCGTCTCGACAATGCTAAA	+	+++

* Underlined type indicates mutation sites

** Partial impairment

Figure 3. Infection of CD4-positive SupT1 cells by N-linked glycosylation mutants. Reverse transcriptase activity in cultured supernatants of SupT1 cells infected by wild type (WT), mutant viruses 141 or 197 were followed for 28 days. The growth kinetics of mutants 88, 162 and 276 were similar to those of mutant 141. The growth kinetics of mutant 262 was similar to those of mutant 197. The growth kinetics of other first-site N-linked glycosylation mutants were similar to those of the wild type virus.



SECTION 2. Comparison of N-linked Glycosylation Sites at the C-terminal of HIV-1 gp120 on Envelope Processing and Viral Infectivity

In the previous study (SECTION 1), we showed that six N-linked glycosylation single site mutants (88, 141, 160, 197, 262, and 276) in the N-terminal half of gp120 affect viral infectivity (16). A model made by Leonard *et al* showed that the cys 131-cys 196 form a loop which has six N-linked glycosylation sites (10) (Figure 1). The C-terminal of gp120 contains CD4 binding regions (8, 39) and several N-linked glycosylation sites. In this study, we generated N-linked glycosylation site combination mutants in these two regions and compared their effects on envelope processing and viral infectivity.

Mutants were generated by oligodeoxynucleotide directed mutagenesis (24). The 2.7 Kb SalI-BamHI fragment of the molecular provirus clone HXB2 (1), which covers most of the coding sequence of the envelope gp160 protein, was cloned into bacteriophage M13mp18 at SalI-BamHI sites and was used as the template for mutagenesis. For all of the N-glycosylation mutations, changes were made from the consensus N-linked glycosylation sequence Asn-X-Ser/Thr (N-X-S/T) to either Gln-X-Ser/Thr (Q-X-S/T) or His-X-Ser/Thr (H-X-S/T) (Table 3). Nine mutants were generated, including N-terminal mutants N2 (141/186 with changes at amino acid positions 141 and 186), N3 (141/160/186), N4 (136/141/160/186), and N5 (136/141/156/160/186), and C-terminal mutants C2 (386/463), C3 (397/463), C4 (386/392/397/463), C5 (386/392/397/406/463), and C6 (386/392/397/406/448/463) (Table 3).

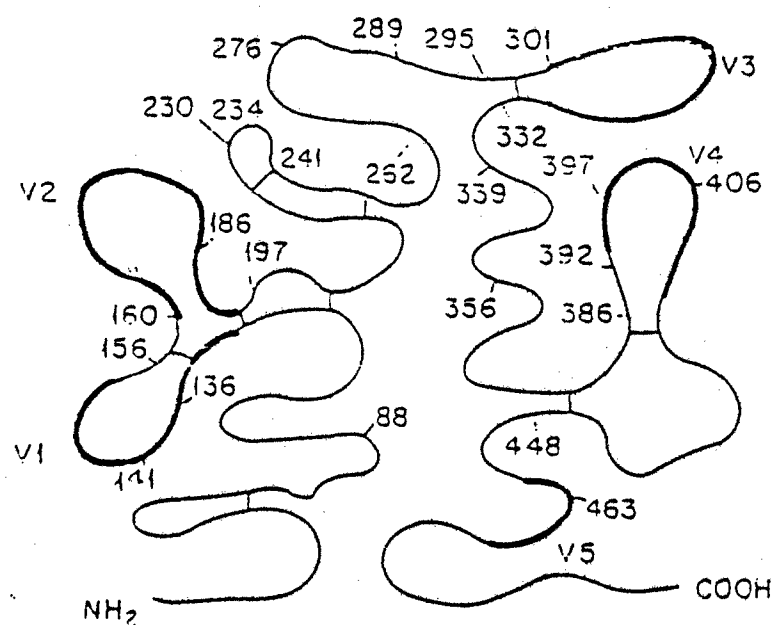
TABLE 3. Combination N-linked glycosylation site mutants of HXB2 *env* glycoprotein

Mutant	Amino acid change	gp160 cleavage	Viral infectivity
C2	386/463	+	+
C3	397/463	+	+
C4	386/397/406/463	+	+
C5	386/392/397/406/463	+	+
C6	386/392/397/406/448/463	+	+
N2	141/186	+	+
N3	141/160/186	++	-
N4	141/156/160/186	-	-
N5	141/136/156/160/186	-	-

The mutations were confirmed by Sanger sequencing (27). In order to avoid generating undesired second site mutations during the mutagenesis process, a 0.5 Kb KpnI-StuI fragment of the mutants construct, which spans the N-terminal mutations, and a 0.58 Kb BglII-BglII mutant DNA fragment, which covers the C-terminal mutations, were swapped with the corresponding fragments

Figure 4.

gpl20 N-LINKED GLYCOSYLATION SITES



of the wild type sequence in pGEM 7Zf(+)-HXB2(EcoRI-BamHI), which contains the 2.7 kB EcoRI-BamHI fragment of gp160. Subsequently, the SalI-BamHI fragment from pGEM 7Zf(+)-HXB2(EcoRI-BamHI) was backcloned into the provirus pSP65HXB2. This assures that the majority of the backbone will contain wild type sequences, and no undesired second site mutations, with the possible exception of the exchanged mutant fragments.

Mutant proviral DNA and wild type DNA (3 µg) were transfected into 3×10^6 COS-7 cells (a monkey kidney cell line, CV-1, origin minus, SV40) by DEAE-dextran method (35). Cell lysates from COS-7 transfected cells collected 48 hours after transfection were used in Western blots. Cell lysate proteins were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with sheep anti-gp120 antisera (4) (Figure 5). The results show that both wild type and mutants N2, C2, C3, C4, C5, and C6 have distinct gp160 and gp120 bands in similar ratios; therefore, we conclude that those mutants have no apparent effect on gp160 cleavage to gp120 and gp41. For mutant N3, the ratio of gp160 to gp120 is higher than that of the wild type suggesting inefficient envelope processing. Mutants N4 and N5 had gp160 without gp120, suggesting that envelope processing was blocked. However, the mobilities of these proteins in the mutants seems to be higher than those of the wild type (Figure 5). Given that previous biochemical and genetic data shows that potential N-linked glycosylation sites are utilized in gp120 (16, 17), this observation strongly suggests that some carbohydrates have been removed from these mutants. This removal accounts for the apparent increases in mobility of gp160 and gp120.

In order to test the effect of the removal of carbohydrates from the C-terminal of gp120 on viral infectivity, cell free virus obtained from these mutants was used to infect CD4-positive T cell line, SupT1. Supernatants were collected from COS-7 transfected cells 48 hours post-transfection. Reverse transcriptase (RT) assays were performed to standardize the amount of input virus for subsequent infection experiments. Virus loads adjusted to RT activities of approximately 400K cpm were used to infect 4×10^6 SupT1 cells. The infectivity of the mutant and the wild type viruses was determined by syncytium formation and RT assays (Table 3). RT activity was measured by mixing 1 ml medium supernatant containing the released virus with poly(rA), oligo(dT), and ^3H -TTP as described (16). The wild type and mutants C2, C3, C4, C5, and C6 all began to form syncytium around day 4 postinfection and had similar kinetics (Figure 6A). This data suggests that carbohydrates at the C-terminal of gp120, which encompasses CD4 binding, are not essential to viral infectivity, directly or indirectly.

The same approach was used to address the effect of the N-linked glycosylation sites on the cys131-196 loop. Mutant N2 formed syncytium at day 4 postinfection and had a much higher RT output than that of the wild type (Figure 6B). In contrast, removal of more than three N-linked glycosylation sites (mutants N3, N4, and N5) in the N-terminal of gp120 did affect viral infectivity

Figure 5. Envelope glycoproteins of wild type and all mutants. COS-7 cell lysates were prepared 48 hours post-transfection and electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose, and reacted with sheep anti-gp120 antisera. (A) Mock, wild type, C2, C3, C4, C5, and C6. (B) Mock, Wild type, N2, N3, N4, and N5. The positions of gp160 and gp120 are indicated with dashes.

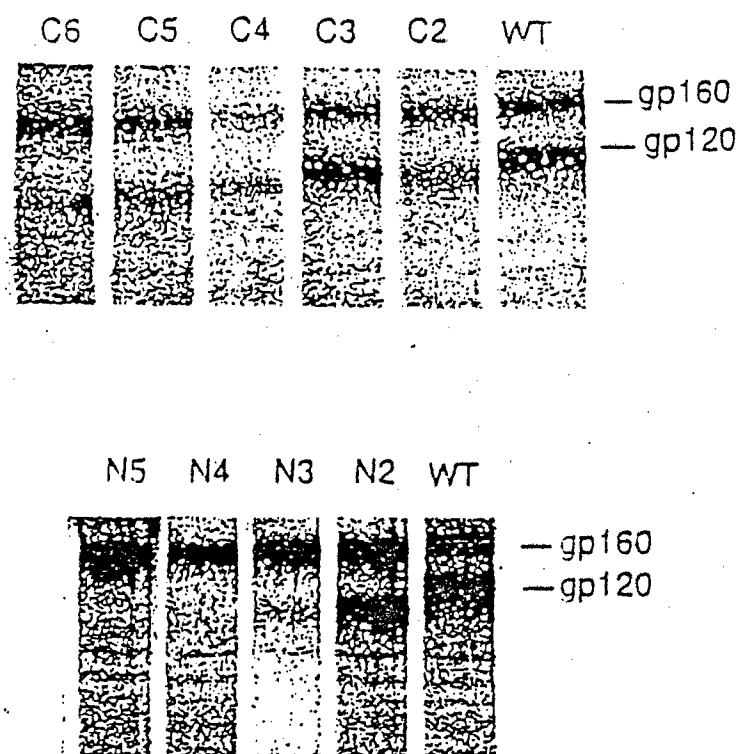
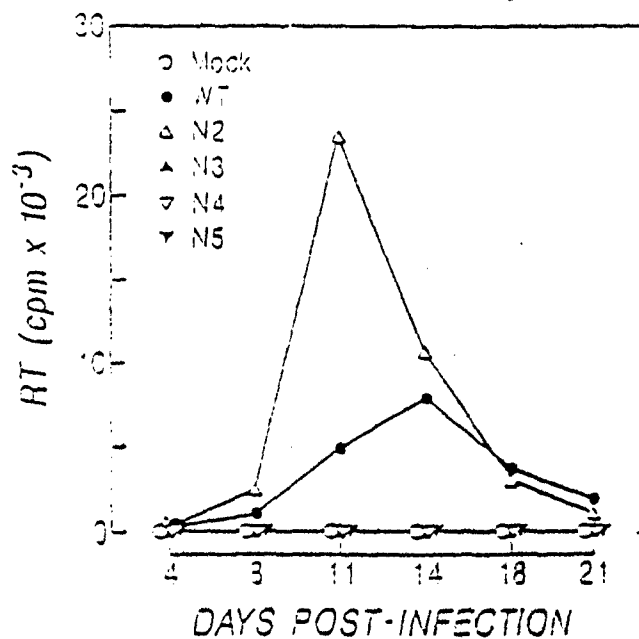
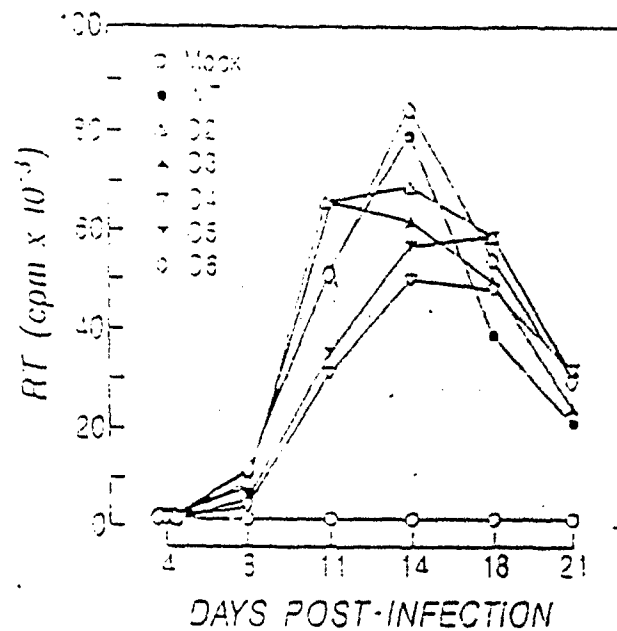


Figure 6. RT activity of CD4-positive T cell line, SupT1 infection by wild type and mutants.
 (A) Mock, wild type. (B) C6 mutant.



in that no syncytia were seen during the observation period. We concluded that at least three N-linked glycosylation sites (141, 160, and 186) at the cys131-196 loop are critical for viral infectivity. A priori, the impairment of viral infectivity of mutants N3, N4, and N5 and the much higher RT output of mutant N2 in the supernatant can be explained by either the removal of carbohydrates or by the substitution of amino acids. In order to further differentiate between these two possibilities, we generated the two mutants N2' and N3'. N2' (143/188) is similar to N2 but replaces Ser/Thr of the consensus N-linked glycosylation site sequence Asn-X-ser/Thr with Ala/Val. N3' (143/162/188) was similarly altered. Mutant N2' had some defect in envelope processing (Figure 8) and a slight delay in RT kinetics (Figure 6C). However, mutant N3' showed a similar phenotype to mutant N3 in its defect in envelope processing (Figure 8) and its infection of SupT1 cells (Figure 6B). This data suggests that the impairment of envelope processing and the defect in virus infectivity of mutant N3 can be attributed to the removal of carbohydrate rather than the substitution of amino acids. However, the higher RT output of mutant N2 is not due to the removal of carbohydrates, but is due to the amino acid change. The slower RT kinetic of mutant N2' may also result from the conformational change due to amino acid substitution.

Since mutant N2 had very high RT output, we looked at the difference in the infected cells which might cause this phenotype. Supernatants and cells collected from wild type, N-terminus mutants N2, and N2', and C-terminus mutants C2, C3, C4, C5, and C6-infected SupT1 cell cultures were fixed by electron microscopy fixing medium and examined under electron microscopy (EM). The syncytia of the mutant N2' (data not shown) and all C-terminus mutants are similar to those of the wild type under EM. (Figure 7A, 7B). However, significant enhancement of virus budding on the surface of cells was observed with the mutant N2 (Figure 7C). High RT output of the mutant N2 in the supernatants may be the result of an increasing amount of virus that was released.

Previous experiments have shown that viral infectivity is severely diminished following binding of lentil lectins to mannose-containing carbohydrate moieties on the HIV-1 viral envelope glycoprotein (18). Hypotheses used to explain the lectin blocking experiments include lectin interfering directly with CD4-gp120 interaction, protein-protein interaction by steric hindrance, and conformational change upon binding. We tested these possibilities with the C-terminus mutant C6 which lacks the six carbohydrates around the CD4 binding region. To evaluate the effect of lentil lectin on free virus infectivity, the virus containing 10^5 cpm RT activity virus equivalent was pretreated with lentil lectin (50 μ g/ml), or medium for 30 minutes at 37°C. This pretreated virus was incubated with 4×10^6 SupT1 cells at 37°C for 1 hour. The cells were then washed and resuspended to a density of 10^6 cells/ml, with lentil lectin added to 50 μ g/ml. The culture was followed for 16 days. Syncytium formation was observed by light microscope. RT activity of the supernatants was measured every three or four days. With the addition of lentil lectin, the

Figure 7. Electron micrographs of wild type, N2, N2', and C6 mutant-infected SuprT1 cells. (A) wild type. (B) C6 mutant. (C) N2 mutant.

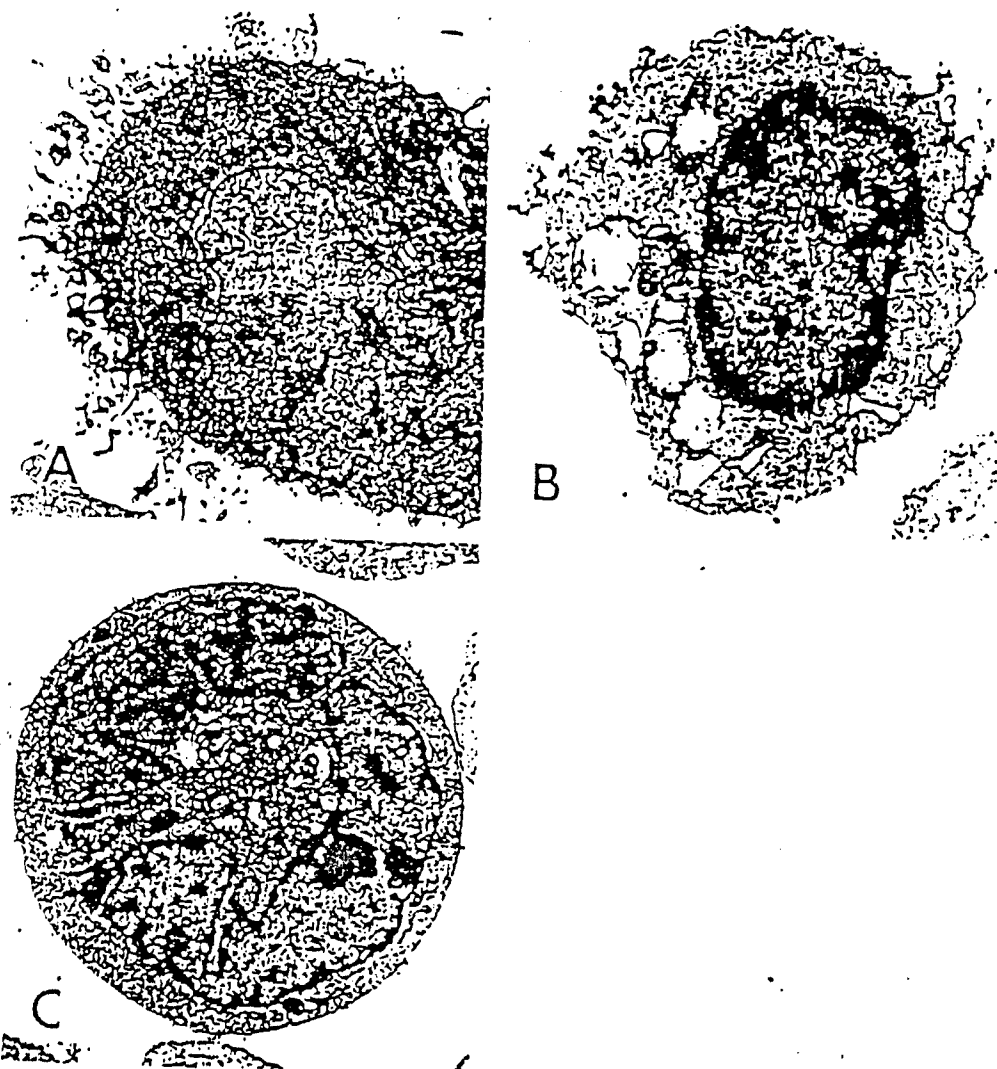
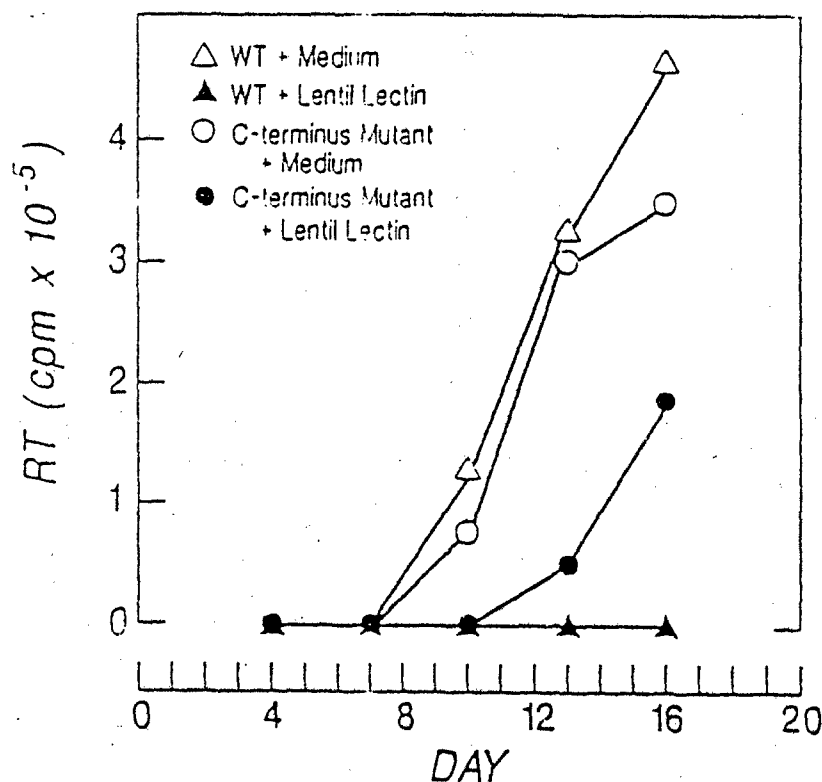


Figure 8. Lentil lectin inhibits HIV virion infectivity for SupT1 cells.



infectivity of the wild type virus can be blocked for at least 16 days postinfection as scored by syncytium formation and RT kinetics; however, with the C6 virus the blockage is incomplete (Figure 5). Syncytia can be observed as early as day 10 postinfection with RT beginning to rise around the same time. This incomplete blockage of mutant C6 implies that the carbohydrates in the CD4 binding region may be responsible for the difference in the degree of blockage between the wild type and mutant C6. Mutant N2 was used to test whether carbohydrates at position 141 and 186 are responsible for the blockage. Both mutant N2 and the wild type can be completely blocked (data not shown). This suggests that two oligosaccharides attached at these two N-linked glycosylation sites are not critical for the blockage.

CONCLUSION

Our study of single-site mutants, in which either the asparagine or the serine/threonine residue of the canonical Asn-X-Ser/Thr N-linked glycosylation sequences was replaced by a non-canonical sequence, demonstrated that most of the individual N-linked glycosylation sites of HXB2 were dispensable for viral infectivity. This is surprising in view of the high degree of conservation of these N-linked glycosylation sites (37). One possible explanation for this unexpected observation is that the removal of a single N-linked glycosylation site is not sufficient to abolish the infectivity of these mutant viruses. Another possibility is that N-linked sugars are not present at most of the conserved N-linked glycosylation sequences, thus viral infectivity would be less likely to be abolished by the introduced point mutations. The latter possibility is unlikely because unusually heavy oligosaccharide structures have not been found in the heavily glycosylated gp120 or gp160 (8,24). Additionally, our observation that most of the mutants had discernible changes in the migration pattern of gp160 and gp120 indirectly supported the notion that N-linked sugars were likely to be present at most of these N-linked glycosylation sites.

An alternative explanation for the dispensable nature of most of the individual N-linked glycosylation sites is that some of these N-linked glycosylation sites may have been conserved by HIV-1 for the purpose of evading host immune response. Previous studies have shown that N-linked sugars can serve as immune decoys and/or have a role in masking neutralizing epitopes of the envelope glycoproteins of two other animal retroviruses (44,45). Under this hypothesis, the removal of such N-linked glycosylation sites would not be expected to affect viral infectivity because they are not conserved to maintain the structural integrity of the envelope protein.

While none of the N-linked glycosylation mutants lost viral infectivity completely, our results did suggest that N-linked sugars at five of the 24 N-linked glycosylation sites might be important for viral infectivity. These sites are represented by mutants 88 or 90, 141 or 143, 197 or 199, 262 or 264, and 276 or 278 (Fig. 1). Among them, the ones represented by mutants 88 or 90, and 262 or 264 are located outside the hypervariable regions of gp120 (1,10,11,29,34,37). The ones represented by mutants 197 or 199 and 276 or 278 are located adjacent to hypervariable regions. The N-linked glycosylation site represented by mutants 141 or 143 is located within a hypervariable region. Our finding that the N-linked glycosylation site represented by mutants 141 or 143 is important for viral infectivity may explain why this N-linked glycosylation site is conserved by most HIV-1 isolates even though it is located within a hypervariable region. At present, we do not have direct evidence for the presence of N-linked sugars at these five sites. However, because viral infectivity was impaired when either the asparagine or the serine/threonine residue of the canonical N-linked glycosylation sequence was replaced by a non-canonical residue at these five sites, the possibility that the impairment of viral infectivity was caused, at least in part,

by the loss of N-linked sugars should be considered. By contrast, N-linked sugars at the N-linked glycosylation site represented by mutants 160 or 162 was unlikely to have a role in the observed defect in infectivity for mutant 160, because mutant 162 and the wild type had a similar phenotype.

Previously, Willey et al. studied four gp120 N-linked glycosylation site mutants derived from another HIV-1 provirus clone, pNL4-3, which shares a high degree of sequence homology in the envelope coding region with HXB2 (34). The four N-linked glycosylation sites altered in that study were represented by mutants 6992, 7055, 7099, and 7136, which were represented by our mutants 241, 262 or 264, 276 or 278, and 289, respectively. Three of the four mutants had a phenotype similar to that of the wild type virus and only mutant 7055 (our mutant 262) had severe impairment in infectivity. The loss of infectivity by this mutant was attributed to amino acid substitutions rather than the loss of this particular N-linked glycosylation site per se (34). This conclusion was based on observations that amino acid substitutions introduced to the X residue (a glycine residue in pNL4-3) of the canonical Asn-X-Ser/Thr sequence (mutant 7058) and to the residue proximal to the asparagine residue of the canonical Asn-X-Ser/Thr sequence (mutant 7052), caused impairment in viral infectivity (34). Because amino acid substitutions were not introduced to replace the serine residue of the canonical Asn-X-Ser/Thr sequence with a non-canonical residue in that study and there was no indication that such mutants, if generated, would have the same phenotype as the wild type virus, the possibility that the N-linked glycosylation site represented by mutant 7055 is critical for viral infectivity remains open.

Because previous studies have demonstrated that the complex type of N-linked sugars did not have important roles in HIV-1 infectivity (40,42), our finding that N-linked glycosylation sites important for HIV-1 infectivity were those distributed in the N-terminal half of the gp120 molecule was unexpected. Previously, Pollack and Atkinson reported that the complex type of N-linked sugars were generally located towards the N-terminus of membrane-bound glycoproteins and suggested that the position of the glycosylation site with respect to the N-terminus affected the extent of oligosaccharide processing and subsequent presentation of complex or high mannose structures in the mature glycoprotein (46). It is conceivable that, at some N-linked glycosylation sites in the N-terminal region of gp120, the incompletely processed precursors of the complex type of N-linked sugars are sufficient to play a crucial role in maintaining the required gp120 conformation for HIV-1 infectivity. By altering the canonical N-linked glycosylation sequence, our genetic approach precluded altogether the transfer of the precursor core of the N-linked sugars to the nascent envelope protein, which may contribute to the impairment of viral infectivity.

The findings of the present study suggest that consensus N-linked glycosylation sites which are important for viral infectivity are not randomly distributed in the gp120 molecule. More recently, Bolmstedt et al reported that the removal of N-linked glycosylation sites represented by our mutants 406 or 463 from the envelope recombinant proteins expressed by a vaccinia

expression vector did not affect CD4 receptor-binding or syncytium-formation (47). Their results were compatible with our finding that CD4-positive SupT⁺ cells were readily infected by our mutants 406 and 463, and support our hypothesis that the N-linked glycosylation sites located in the C-terminal half of gp120 are more dispensable than those located in the N-terminal half of gp120 for viral infectivity. If our hypothesis that some of the dispensable N-linked glycosylation sites are conserved by HIV-1 to evade host immune response is correct, a partially glycosylated gp120 with most of these sites removed may represent a better vaccine candidate than the fully glycosylated gp120.

We showed that six N-linked glycosylation sites at the C-terminal of gp120 covering the CD4 binding region did not affect cleavage of gp160 to gp120 and viral infectivity. The size of gp160/gp120 of mutants C2, C3, C4, C5, and C6 are smaller than those of the wild type. This suggests that there are carbohydrates attached to these N-linked glycosylation sites. We cannot rule out that changing amino acids affects protein folding and leads to the changing of mobility of gp160/gp120; however, the obvious size difference between the mutants and wild type cannot simply be due to the amino acid substitution. If indeed the gp160/gp120 mutant size decrease is due to the removal of carbohydrates, the N-linked oligosaccharides at the C-terminal of gp120 are not critical for viral infectivity. The binding of gp120 to CD4 is required to infect CD4-positive T cells. Our results suggested that carbohydrates covering the CD4 binding region are not important to gp120/CD4 interaction. It has been shown that mannose-binding lectins inhibit virus infection (18). Our data showed that the carbohydrates in the CD4 binding region are responsible for the difference in the degree of lentil lectin blockage between wild type and mutant C6. The carbohydrates removed in mutant C6 may bind to lentil lectin then affect CD4-gp120 interaction or may be responsible for interference of protein/protein interactions through steric hindrance. The other possibility is that the removal of these carbohydrates may cause conformation change in the structure of the envelope glycoprotein upon binding.

For mutants N2, N2', N3, N3', N4, and N5, the size of gp160/gp120 is smaller than that of the wild type. This suggests that the mutation introduced at these N-linked glycosylation sites indeed causes the removal of carbohydrates. Mutant N2 had very high RT output compared to the wild type. This high RT output may be caused by increased release of the virus through crowded budding of the virus from the surface. This suggested that envelope mutation may affect virus release. However, this is not due to the removal of carbohydrates, since mutant N2' lacks the same carbohydrates and did not give the same phenotype. The amino acid substitution at positions 141 and 186 result in the high RT output of mutant N2. Mutants N3, N3', N4, and N5 lost the ability to infect SupT1 cells. The inefficient envelope processing of N3 and N3', and the lack of cleavage of gp160 to gp120 of N4 and N5 could cause the loss of viral infectivity. It is understood

that this phenotype can be explained equally well by conformational changes introduced either by the removal of carbohydrates or by the substitution of amino acids. Since mutant N3' showed the same phenotype as mutant N3, it supported the removal of carbohydrates affects viral infectivity. Certainly, the amino acid substitution hypothesis remains very much viable, and a combination of the two possibilities cannot be ruled out.

N-linked carbohydrates can have many biologic functions such as preventing of proteolytic degradation (22, 31) and intracellular aggregation (5, 9, 19, 20), assisting in protein folding (5, 15, 20, 21, 32), etc. Here we have shown that N-linked glycosylation sites at the N-terminal cys131-196 loop of gp120 are important for envelope processing and viral infectivity. These effects are most likely due to the removal of carbohydrate rather than amino acid substitutions. However, carbohydrate covering the CD4 binding region at the C-terminal of gp120 are not critical for envelope processing and viral infectivity. Low immunogenicity of synthetic peptide containing V4 and V5 sequences upon vaccination (25) and the dispensable feature of the N-linked glycosylation sites at this region suggest that glycosylation at this region may mask the CD4 binding region. The conservation of these N-linked glycosylation sites at C-terminal of gp120 which contains CD4 binding region may be an evolutionary response for HIV to escape immune surveillance. For future vaccine development, at least some of the N-linked glycosylation sites of the cys 131-196 loop have to remain to ensure that proper conformation is maintained. Furthermore, to generate molecules for the purpose of crystallography, those carbohydrates at the C-terminal of gp120 can be removed while the CD4 binding domain remains intact. From our results, the lentil lectin blocking region not only in the those mannose-containing carbohydrates moieties covering the CD4 binding region of C-terminal of gp120, but also the carbohydrates outside this region. All of this information may be helpful for the development of antiviral drugs.

To summarize the principal findings from this report: (1) We have demonstrated that most of the N-CHO sites on gp120 are most likely glycosylated. (2) We have identified several N-linked oligosaccharides on gp120 which may be critical for viral infectivity. These findings suggest that some N-CHO sites may be potential targets for inhibitors. (3) We found that some N-CHO sites at the C-terminus of gp120 are dispensable for several biological functions in vitro, in particular, envelope processing, syncytium formation and viral infectivity. This implies that N-linked carbohydrates at the putative CD4 binding region are not critically involved in CD4-gp120 interaction. One difficulty faced in the crystallization of gp120 is its heavily glycosylated feature. Since our data indicate that at least six N-CHO sites are not required for forming an important biological functional conformation, the C6 mutant may be a better molecule for crystallization.

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